Peptide-mediated cell delivery: application in protein target validation

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Recent reports have suggested that conjugation of peptides, proteins and antisense to short highly basic peptides, such as TAT, antennapedia and transportan, results in their rapid translocation into cells. Importantly, these conjugates have been shown to exert actions in a number of animal models suggesting their general utility for the determination of protein function *in vitro* and *in vivo*.

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Abbreviations
PNA   peptide nucleic acid
PTD   protein transduction domain

Introduction

The complexity of biological interactions makes it increasingly difficult to predict gene and protein function as you proceed from the immediate metabolic pathway to the cellular and animal level. This phenomenon is partially responsible for the high attrition rate observed during drug development and necessitates early validation of protein function in cellular and/or animal models that are predicative of the disease. Classically, this is undertaken either through acute modulation using protein knockdown and/or inhibition, or using chronic models, such as transgenic and knockout mice.

In cellular systems, a variety of tools are employed to determine protein function, including antisense, peptide modulators and the overexpression of wild-type or dominant-negative protein. However, these studies are often limited by the inability to effectively deliver these validation tools. Typically, delivery is via lipids, electroporation or through viral vectors, but these have several severe limitations, including the inability to deliver to primary, non-dividing cells, the requirement for optimisation with each cell type, low transfection levels and cellular toxicity. Interestingly, recent studies have identified several short peptide sequences named protein transduction domains (PTDs) or cell penetrating peptides (CPPs), which appear to rapidly translocate into all cells both *in vitro* and *in vivo*. Importantly, conjugation of proteins, peptides and antisense to these PTDs has been shown to deliver these cargos effectively, allowing observation of biological action in several cell and animal models [1,2]. In this review, we examine the use of PTDs as a novel and potentially universal delivery system for delineation of protein function and target validation.

Peptide transduction domains

PTDs were first identified while investigating the spontaneous cell entry of HIV TAT, which is subsequently localised to the nucleus and transactivates the viral long-terminal repeat promotor encoded within the human immunodefficiency virus [3]. Studies of the minimum translocation region identified a positively charged section between amino acids 47 and 57, which was previously associated with DNA binding [4]. Similar studies of antennapedia, a *Drosophila* homeodomain transcription factor, identified a 16-amino-acid PTD derived from region 43–58, and also located within the DNA-binding third domain [5–7]. Since these initial observations, a host of short peptides have been identified and shown to rapidly translocate across membranes. However, conjugation and delivery of biological cargos has been predominantly performed using the peptides derived from TAT (sequence, in single-letter amino acid code: YGRKKRRQRRR), antennapedia (RQIKIWFQN-RRMKWKK) and transportan (GWTLNSAGYLLGPH-IDNHRSFHDKYGLA), a synthetic chimera derived from galanin and mastoparan [8].

Mechanism of cell entry

To date, studies of the mechanism of cell entry have largely examined the movement of labelled antennapedia and TAT across artificial membrane systems or into cells. Although this pathway is probably analogous to the situation during PTD-mediated delivery of small peptide cargos, it is likely that the mechanism will be different with larger macromolecules such as antisense and proteins. With this caveat in mind, the studies have identified several important characteristics, outlined below.

Dependence on basic arginine residues

Substitution of alanine for arginine in the TAT sequence significantly attenuated translocation, whereas simple polyarginine sequences, with an optimal length of 8–9 mer, produced uptake rates that were significantly higher than TAT [9,10]. The guanidine headgroup is the critical structural feature, as homopolymers of citrulline, an arginine isostere, show no transduction, whereas increasing the distance between the guanidine group and the peptide backbone, using alkyl spacers, further increased uptake [11].

Independence of the endocytic pathway
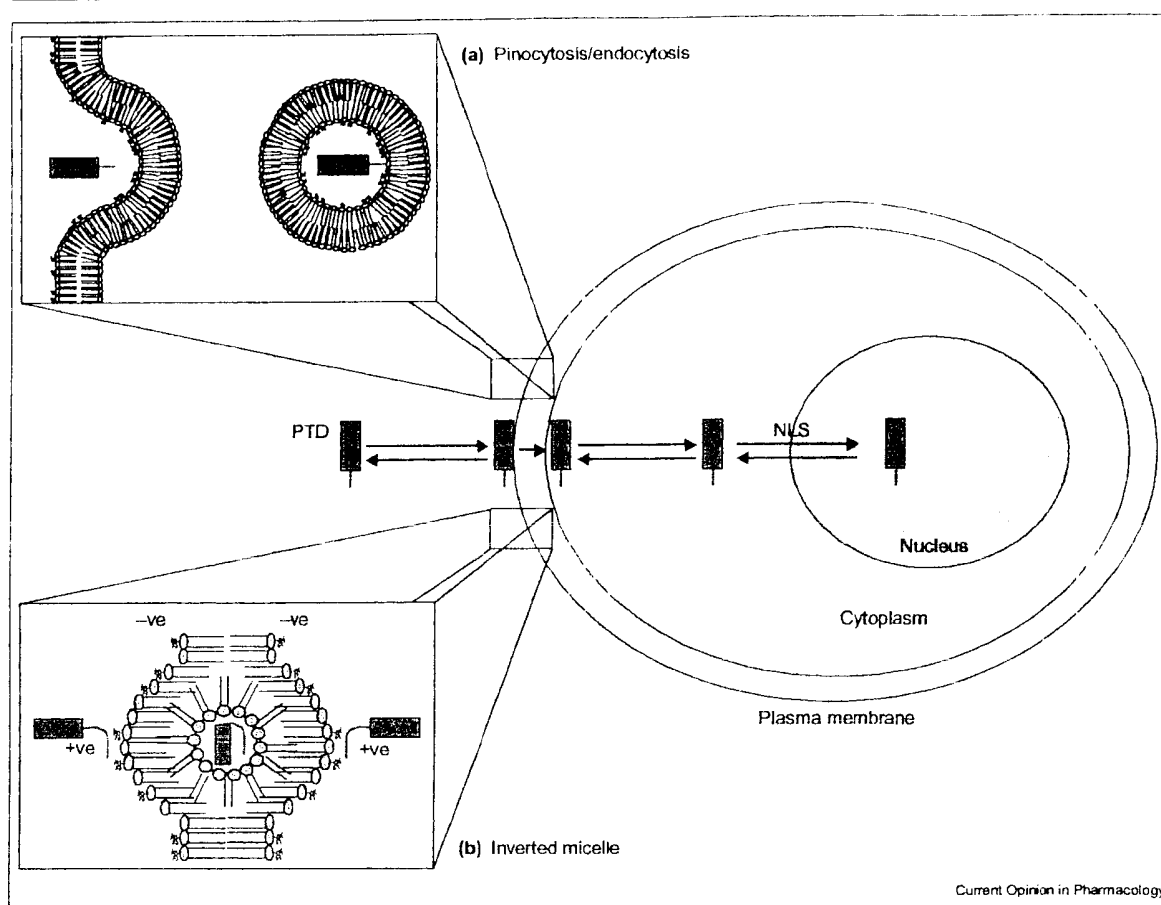
Uptake is unaffected by a range of endocytosis inhibitors, including wortmannin, cytochalasin D, colchicines, nocodazole, taxol, brefeldin A and chloroquine, and is only partially attenuated when the temperature is reduced from 37°C to 4°C [4,5,12].

Energy-independent mechanism

Uptake is unaffected by metabolic inhibitors, including sodium azide and rotenone [4,5,12].

Figure 1

(a) Pinocytosis/endocytosis

PTD   NLS

Nucleus

Cytoplasm

Plasma membrane

(b) Inverted micelle

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Mechanism of PTD-mediated delivery. Initial binding is mediated through electrostatic interaction between positively charged (+ve) PTD sequences (purple line) and biological cargo (red box) and negatively charged (–ve) membrane phospholipids (grey circles). Little is known of the subsequent cellular uptake mechanism, although delivery by both (a) an as yet undefined pinocytosis/endocytosis pathway and (b) inverted-micelle-mediated membrane shuttling produced by the interaction of hydrophobic amino acids and the membrane has been proposed. Once released into the cytoplasm, PTD-conjugates can be targeted to the nucleus using nuclear translocation sequences (NLS).

Receptor-independent mechanism

Delivery of antennapedia [5,13] and TAT [10,11] is unaffected by the retro-inverso sequence or when these PTDs are composed entirely of D-enantiomers, indicating that the process is not dependent on receptors.

The identification of micelles during nuclear magnetic resonance studies of antennapedia translocation across phospholipid membranes [14], prompted Alain Prochiantz to propose an inverted micelle model to explain antennapedia translocation (Figure 1). This envisages initial electrostatic interaction between the peptide (positive charge) and membrane phospholipid (negative charge) followed by plasma membrane shuttling, through formation of unil-amellar phospholipid bubbles. However, inverted micelle formation requires the presence of hydrophobic amino acids that are absent in TAT and other PTDs, implying

that this is unlikely to represent the common PTD delivery mechanism. Alternatively, although there are no studies to date that have addressed this issue, it would appear more likely that PTD delivery of proteins and antisense is mediated via an as yet uncharacterised pinocytosis/endocytosis-related mechanism (Figure 1).

Central to using PTDs is not only their ability to deliver tools but, importantly, to deliver tools at concentrations and for time periods sufficient to give biological activity. Comparison of the uptake rate by fluorescence resonance energy transfer (FRET) analysis showed that the transportan uptake rate was greater then TAT, which was greater than antennapedia and, like many other studies using labelled PTD, that uptake was essentially complete within 15–60 min [15•]. Examination of intracellular PTD levels showed that these are correlated with the rate of

Table 1

PTD-mediated peptide delivery.

Target	PTD	Cell type	Cellular response	Ref
IL-4 signalling (SH2 domain)	Ant	RAMOS, Caki-1, PBL and HeLa	Inhibition of Stat6 phosphorylation	[35]
β_1 integrin	TAT	Neutrophils	Inhibition of PI3K and transmatrix migration	[36]
JNK-1/2	TAT	TC3	IL-1β induced JNK activation and apoptosis	[37]
p53-mdm2 binding	Ant	Cancer cell lines including BMRPA1, E49 and HeLa	Induced apoptosis	[38]
BAD (BH3 domain)	Ant	HeLa, CEM and 32D cells	Apoptosis induction	[39]
NADPH oxidase	TAT	In vivo (rat)	Inhibition of angiotensin-induced O_2^- levels in aortic rings and systolic blood pressure	[33]
IKK2	Ant	HeLa and in vivo (mouse)	Inhibition of NEMO (IKK3) induced IKK2 activation in vitro and acute inflammatory response in vivo	[34]
G1 cyclin-Cdk complex	Ant	AsPC-1 and BxPC-3	p16^{INK4A}-derived peptide attenuated cell proliferation	[40]
cGMP-dependent protein kinase 1α	TAT Ant	In vivo (rat)	NO-induced cerebral dilutation in rat cerebral arteries	[41]
PDGF/IGF/insulin signalling (SH3 domain)	Ant	NIH3T3	DNA synthesis and proliferation	[42]
PKC-RACK binding	Ant	Ex vivo (rabbit adult ventricular myocytes)	Abolished the protection derived from ischaemic pre-conditioning	[43]
Grb2-Sos binding during NGF/EGF signalling (SH3 domain)	Ant	ER22/PC12/NIH3T3/HER2	Grb2-Sos binding, ERK-1/2 activation and proliferation	[44]
Bak (BH3 domain)	Ant	HeLa cells	Inhibited apoptosis	[45]
Cdk2/4	Ant	CA46	p21$^{Waf1/Cip1}$-derived peptide attenuated cell growth	[46]
Inhibition of GRB10-receptor binding (SH2/SH3 domain)	Ant	NIH3T3 cells	DNA synthesis and proliferation	[47]
PI3K (SH2 domain)	Ant	Cerebellar granule cells	Apoptosis	[48]
c-Myc-DNA binding	Ant	MCF-7	Cell growth/apoptosis and ODC/p53 regulation	[49]
Cdk4/6	Ant	HaCal cells	p16^{INK4A}-derived peptide attenuated cell growth	[18]
CD44 cytoplasmic domain	Ant	RPM-MC	Cell migration	[50]
Phosphatidylinositol 3-kinase (SH2 domain)	Ant	C2 muscle cell	Stimulates entry into S-phase	[51]
Cdk2/4	Ant	SKOV-3/IGROV-1	p21$^{Waf1/Cip1}$-derived peptide attenuated Cdk activity and cell proliferation	[52]
EGF/PDGF signalling (SH2 domain)	Ant	Newt A1 myoblasts and mammalian C2 myoblast cells	Proliferation and ERK-1/2 activation	[53]
FGF signalling (SH2 domain)	Ant	Rat small cerebellar neurons	PLCγ activation and neurite outgrowth	[19]
EGF signalling (SH2 domain)	Ant	SAA cells	Ras and ERK-1/2 activation	[54]

Ant, Antennapedia; CdK, cyclin-dependent kinase; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; JNK, c-Jun N-terminal kinase; IGF, insulin-like growth factor; IKK, IκB kinase; IL, interleukin; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol-3-kinase; PKC, protein kinase C; PLC, phospholipase C; RACK, receptor for activated C kinase.

uptake and, interestingly, that these are significantly higher than those in the extracellular media. Thus, incubation for 60 min with 5 μM labelled peptide gave intracellular concentrations of 1800 μM, 500 μM and 350 μM for transportan, TAT and antennapedia, respectively [15*], an observation confirmed by high-performance liquid chromatography determination of antennapedia delivery [16]. Interestingly, it also appears that the uptake level is comparable across different cell types and is independent of the PTD, implying that they employ similar delivery mechanisms [12,16].

Peptide delivery

Both antennapedia and TAT have been used in peptide delivery and have been effectively employed to attenuate protein–protein and enzyme–substrate interactions involved in growth factor, cytokine and integrin signalling,

apoptosis, and cell division (Table 1; [17••]). Examination of the literature shows that, compared with PTD–protein conjugates, substantially greater concentrations of PTD–peptide conjugates (>10 μM) were required for biological activity. The proteins are likely to have a greater affinity, resulting from their secondary and tertiary structure. This was clearly shown in a study by Fahraeus et al. [18], who found that the full-length protein and a short inhibitory peptide sequence derived from p16 INK4A, gave IC$_{50}$s of 10 nM and 10 μM, respectively, when measuring inhibition of the kinase cdk4/6. It thus appears that the most suitable peptide modulators for this approach would be short in length and require no secondary and tertiary structure for specificity and binding. As an example, PTD-mediated delivery of phosphotyrosines has been successfully employed to attenuate binding through SH2 domains. This inhibition is often very selective because

Table 2

PTD-mediated protein delivery.

Protein	PTD	Cell type	Cellular response	Ref
APO-BEC-1	TAT	Primary hepatocytes	Induced apoB mRNA editing and enhanced synthesis/secretion of VLDL	[55]
Cre recombinase	TAT	CV-1 fibroblasts, mouse embryonic stem cells and splenocytes	Cre recombination of loxP sites	[32]
Catalase	TAT	PC12 and mouse skin	Cell viability following oxidative stress	[24]
RhoA	TAT	Human eosinophils	Migration	[56]
H-Ras	TAT	Human eosinophils	IL-5 induced ERK-1/2 activation, p90 Rsk1 phosphorylation and survival	[57]
Filamin-1	TAT	HEK-293	ERK-1/2 activation	[58]
IκBα	TAT	Mouse bone marrow macrophages and osteoclasts	Osteoclastogenesis	[59]
β-Galactosidase/Bcl-X$_L$/PEA-15	TAT	Rat pancreatic islets (β-cells)	Apoptosis	[60]
p16	TAT	Primary human diploid fibroblasts	G$_1$ cyclin–Cdk complexes	[61]
HPC-1/syntaxin	TAT	PC12	K$^+$ and carbachol induced noradrenaline and dopamine release	[62]
Cdk2	TAT	Human mammary epithelial cells	Cell division progression via the $\alpha_v\beta_3$ integrin	[63]
Rho/Rac/Cdc42	TAT	Human dermal microvascular endothelial cells	VEGF-stimulated endothelial cell motility	[64]
RhoA	TAT	Avian osteoclast	PI3K activation, podosome assembly, stress fibre formation, osteoclast motility and bone resorption	[65]
Superoxide dismutase	TAT	HeLa	Oxidative stress	[23]
E2F-1/p73/p53	TAT	Jurkat T-cells/primary T-cells	TCR activation induced cell death	[66]
Caspase-3	TAT	Jurkat T-cells	HIV-protease activation in infected cells cleaved and activated caspase-3 to induce cellular apoptosis	[67]
p16^{INK4A}	Ant	Primary human diploid fibroblasts	Growth arrest	[20]
p27^{Kip1} Cdk inhibitor	TAT	Jurkat T-cells, hepatocellular carcinoma	Cell division and migration	[68]
p16^{INK4A}	TAT	HaCaT/HepG2/Jurkat T-cells	Retinoblastoma phosphorylation	[69]

Cdk, cyclin-dependent kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; IκB, inhibitor of NFκB; IL, interleukin; PI3K, phosphatidylinositol-3-kinase; TCR, T-cell receptor; VEGF, vascular endothelial growth factor; VLDL, very low-density lipoprotein.

the specificity of SH2 binding to a particular phosphotyrosine sequence is determined by the three to five amino acids that are C-terminal to the tyrosine. Thus, a peptide based around a phosphotyrosine site (Tyr766) upon the fibroblast growth factor receptor 1 (FGFR-1) and attached to antennapedia selectively inhibited phosphatidylinositol signalling and subsequent neurite outgrowth in primary neurons, but had no effect upon survival, differentiation or phosphatidylinositol hydrolysis stimulated by platelet-derived growth factor, neurotrophin-3 or bradykinin [19].

Protein delivery

Although antennapedia-mediated [20] and transportan-mediated [21] protein delivery has been reported, Steven Dowdy has pioneered this area using TAT-conjugated proteins [22*]. As with the peptides, this has been successfully employed to deliver several targets, including dominant-negative GTP-binding proteins (Rho, Rac, Cdc42), modulators of cyclin-dependent kinase (cdk) activation (p16^{INK4A} and p27^{KIP1}) and inhibitor of NFκB (IκB)-α (Table 2). Furthermore, as discussed earlier, TAT proteins appear to be considerably more efficacious than peptides, often giving biological activity at submicromolar concentrations.

Few investigators have examined the mechanism of TAT-mediated protein uptake, although recent observations imply that this may differ significantly from that of TAT-mediated peptide uptake. Measurement of catalase and superoxide dismutase (SOD) delivery by TAT, determined by western blotting, showed uptake at 10–30 min, which peaked at 4–6 hours before dropping to basal levels at 48–72 hours [23,24]. However, in contrast to TAT–peptide uptake, TAT–protein uptake (of catalase and SOD) was found to be attenuated at 4°C [25*] and to be dependent on binding to the negatively charged heparin sulfate proteoglycans [12,25*,26]. With a few exceptions, it is also thought that TAT–protein uptake is facilitated when proteins are in a partially re-natured state. For example, SOD and catalase uptake fails to occur when these proteins are prepared in the native state [23,24]. The reason for this phenomenon is unknown, although it has been speculated that the uptake mechanism is driven by the decrease in entropy following protein refolding within the cell. Alternatively, it might also be envisaged that the partially re-natured state might permit more efficient interaction of TAT with the positively charged cellular membrane.

Table 3

PTD-mediated antisense delivery.

Target	PTD-conjugate	Cell type	Cellular response	Ref
PTPσ	Transportan-PNA	Rat islets of Langerhans	Inhibited glucose-induced insulin secretion	[27]
c-myc antigene	Nuclear localisation sequence-PNA	BJAB/HBL2	Inhibited cell cycle and apoptosis	[28]
P-glycoprotein	Ant-ODN, TAT-ODN	NIH3T3	P-glycoprotein expression	[70]
Galanin receptor	Ant-PNA, transportan-PNA	Bowes/rat (*in vivo*)	Decreased galanin expression and inhibited C-fibre stimulation-induced facilitation of the rat flexor reflex	[29]
Amyloid precursor protein	Ant-ODN	Rat cortical neurones	Axon and dendrite outgrowth	[71]

ODN, oligodeoxyribonucleotide; PTPσ, protein tyrosine phosphatase σ.

Antisense delivery

Although a limited number of studies have employed TAT, transportan and antennapedia for delivery of anti-sense, their general utility has been limited by the difficulty in chemically conjugating the PTD peptide and antisense oligonucleotide backbones (Table 3). However, this problem can be resolved through the use of peptide nucleic acid (PNA) antisense, which has been shown to attenuate protein expression and biological activity of a small number of targets, including protein tyrosine phosphatase σ [27], c-myc [28] and the galanin receptor [29]. Interestingly, the attenuation of c-myc expression was through an action of PNA at the transcriptional level [28]. With few publications in this area it is difficult to draw lessons regarding the mechanism or utility of PTD–anti-sense delivery. However, it could potentially provide an extremely useful and general tool because, unlike peptides and proteins, it should be possible to examine the role of unknown genes.

In vivo delivery

To date, only a small number of studies have investigated the *in vivo* utility of PTD-mediated protein delivery. Considerable interest was first generated by a report demonstrating the presence of active TAT–β-galactosidase in all mice tissues (including the brain) at 4–8 h following intraperitoneal injection [30••]. Since then, a study by Jo *et al.* [31••] has demonstrated Cre delivery to both mammalian cells and mouse tissue, using a PTD derived from Kaposi fibroblast growth factor (FGF)-4 [31••]. Paradoxically, a comparable investigation showed that TAT, but not FGF, mediated cellular Cre delivery when combined with a nuclear localisation sequence [32]. Overall, these two studies suggest that PTD-mediated Cre delivery could be of enormous potential for the conditional knockout of loxP-flanked genes both *in vitro* and *in vivo*. Interestingly, a recent study had shown that following topical application of TAT–catalase to mouse skin, activity was found to penetrate the epidermis as well as the dermis of the subcutaneous layer [24] suggesting that it may be possible to selectively target PTD cargos.

Like PTD-mediated protein delivery, only a few investigations have tackled the use of PTD-mediated peptide delivery, and these have focused on delivery to inhibit *in vivo* function. Thus, peptide inhibitors conjugated to PTD designed to attenuate the activation of NADPH oxidase and IκB kinase 2 (IKK2) have been shown to inhibit rat-angiotensin-induced increases in systolic blood pressure in mice [33] and acute mouse inflammatory responses [34], respectively. Similarly, a single study has examined PTD–antisense delivery and showed that transportan-mediated PNA antisense delivery caused downregulation of galanin receptor expression and inhibition of C-fibre stimulation-induced facilitation of the rat flexor reflex [29].

Conclusions

The recent completion of the first draft of the human genome has provided the scientific community with the majority of the basic building blocks responsible for human biology. The next phase, involving the identification of gene function, would be greatly facilitated by the ability to deliver tools that modulate protein function *in vitro* and *in vivo*. To this end, initial studies have suggested that PTDs may provide a valuable, possibly universal delivery tool for the acute *in vitro* and *in vivo* delivery of peptides, proteins and antisense. However, several questions will need to be addressed to define the overall applicability of this technology. Most importantly, although it appears that initial binding is mediated follow-ing electrostatic interaction at the cell membrane, little is known of the mechanism of cellular uptake, trafficking and metabolism, the differences between PTDs and how these are affected following conjugation to proteins and antisense. Hopefully, an understanding of these factors will permit the optimal design of PTD cargos for the intra-cellular targeting of the protein of interest.

Update

Continuing studies on the mechanism of PTD-mediated uptake have confirmed the importance of positively charged amino acids and demonstrated highly efficient delivery of proteins >500 kDa following conjugation to polylysine sequences [72]. Interestingly, a recent study using confocal microscopy identified the presence of labelled PTD–PNA conjugates in cytosolic vesicular compartments and suggested that delivery is mediated via a receptor-dependent endocytotic pathway [73••]. In both publications, uptake was found to be cell-type-dependent [72,73••].

Of particular interest is a recent *in vivo* study in mice, using a TAT–Bcl-xL construct that was found to cross the blood brain barrier and transduce into neurons of various brain regions within 1–2 h of intraperitoneal injection [73**]. Importantly, using a murine model of focal ischemia/reperfusion, treatment with TAT–Bcl-xL was found to decrease cerebral infarction through inhibition of caspase-3 activation and neuronal apoptosis [73**].

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This paper demonstrates, for the first time, the utility of a peptide–protein conjugate for investigating the role of a protein target in an *in vivo* disease model.